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RESEARCH PAPER

Pharmacokinetic–pharmacodynamic modelling of drug-induced QTc interval prolongation in man: prediction from *in vitro* human ether-à-go-go-related gene binding and functional inhibition assays and conscious dog studies

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BACKGROUND AND PURPOSE

Functional measures of human ether-à-go-go-related gene (hERG; $K_v11.1$) channel inhibition have been prioritized as an *in vitro* screening tool for candidate molecules. However, it is unclear how these results can be translated to humans. Here, we explore how data on drug binding and functional inhibition *in vitro* relate to QT prolongation *in vivo*. Using cisapride, sotalol and moxifloxacin as paradigm compounds, we assessed the relationship between drug concentrations, binding, functional measures and *in vivo* effects in preclinical species and humans.

EXPERIMENTAL APPROACH

Pharmacokinetic–pharmacodynamic modelling was used to characterize the drug effects in hERG functional patch clamp, hERG radio-labelled dofetilide displacement experiments and QT interval in conscious dogs. Data were analysed in parallel to identify potential correlations between pharmacological activity *in vitro* and *in vivo*.

KEY RESULTS

An Emax model could not be used due to large variability in the functional patch clamp assay. Dofetilide displacement revealed that binding curves are unrelated to the *in vivo* potency estimates for QTc prolongation in dogs and humans. Mean *in vitro* potency estimates ranged from 99.9 nM for cisapride to 1030 μM for moxifloxacin.

CONCLUSIONS AND IMPLICATIONS

The lack of standardized protocols for *in vitro* assays leads to significant differences in experimental conditions, making the assessment of *in vitro–in vivo* correlations unreliable. Identification of an accurate safety window during the screening of candidate molecules requires a quantitative framework that disentangles system- from drug-specific properties under physiological conditions, enabling translation of the results to humans. Similar considerations will be relevant for the comprehensive *in vitro* pro-arrhythmia assay initiative.

Abbreviations

hERG ($K_v11.1$), human ether-à-go-go-related gene; NPDE, normalized prediction distribution error; PKPD, pharmacokinetic–pharmacodynamic; TdP, Torsade de Pointes

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Tables of Links

TARGETS
$Ca_v1.2$
$K_v11.1$ (hERG)
$Na_v1.8$

LIGANDS	
Astemizole	Penicillin
Cisapride	Sotalol
Dofetilide	Verapamil
MK-499	

These tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander *et al.*, 2015).

Introduction

During the last two decades, a number of drugs have had to undergo labelling revision or market withdrawal due to post-marketing reports of sudden cardiac death linked to Torsade de Pointes (TdP) (Cavero *et al.*, 2000; Haverkamp *et al.*, 2000; Redfern *et al.*, 2003; Shah and Hondeghem, 2005; Thomsen *et al.*, 2006). Despite major efforts to screen compounds for their pro-arrhythmic activity *in vitro*, most compounds still progress into clinical development with an unclear risk of QT interval prolongation in humans (Chen *et al.*, 2006; Ducroq *et al.*, 2007; Gintant, 2008).

From a drug discovery perspective, pharmaceutical research and development has relied on *in vitro* human ether-à-go-go-related gene (hERG also known as $K_v11.1$ channel) assays as a primary screening filter before *in vivo* experimental protocols are used to evaluate QT/QTc interval prolongation in preclinical species. Multiple approaches have been developed to evaluate drug effects on hERG current *in vitro*. Gintant *et al.* (2006) proposed to divide them into two different classes based on whether experimental measures will reflect a direct or indirect effect on the hERG current. Indirect approaches include binding assays, assays measuring ionic flux changes and assays detecting changes in membrane potential. An advantage of binding assays that measure displacement of potent, radiolabelled hERG ligands is that they provide a convenient screening method to detect drug-hERG channel interactions. These systems use intact cells or cell membranes from heterologous expression systems transfected with the hERG channel and potent, radiolabelled hERG ligands such as dofetilide (Diaz *et al.*, 2004), methanesulfonanilide MK-499 (Wang *et al.*, 2003) and astemizole (Chiu *et al.*, 2004). Results obtained with the aforementioned indirect assays are considered to be less sensitive than those reported using direct measures of the hERG current but have the advantage of greater throughput, as compared with functional measures.

More recently, newer techniques have been developed to assess hERG currents in a more direct way (Dubin *et al.*, 2005). However, there is no evidence yet of how the results from these assays correlate with the drug-induced QT-interval prolongation in humans, even when one considers compounds that show no affinity for other ion channels. In fact, regulatory agencies, academic researchers and pharmaceutical companies appear to have recognized the drawbacks of the existing approach for the evaluation of pro-arrhythmic properties based on a predominant focus on the hERG channel. As a result, at the conference of the Cardiac Safety Research Consortium (CSRC)–Health and Environmental Sciences Institute (HESI)–Food and Drug Administration (FDA) held in July 2013, a revision of ICH S7B and possible elimination of ICH-E14 were proposed. The proposal is aimed at shifting the focus from evaluating QT prolongation to evaluating the pro-arrhythmic activity of a compound using a comprehensive *in vitro* pro-arrhythmia assay (CiPA) (Cavero and Holzgrefe, 2015; Sager *et al.*, 2014; Fermini *et al.*, 2016). The approach seems, however, to overlook the importance of a stricter quantitative framework for the translation of *in vitro* findings and in particular of the potential differences between *in vitro* and *in vivo* concentration–effect [pharmacokinetic–pharmacodynamic (PKPD)] relationships.

Here, we evaluated whether a systematic correlation can be found between hERG binding and functional inhibition data *in vitro*. Subsequently, we attempted to assess how binding and functional inhibition data correlate with the underlying concentration–effect relationship *in vivo*, both in nonclinical species (dogs) and healthy human subjects (Chain, Dubois *et al.*, 2013). Reference compounds with known clinical QT prolonging effects were used for the purposes of this evaluation, namely cisapride, sotalol and moxifloxacin. Evidence of such a correlation might support the use of hERG binding data in conjunction with PKPD relationships as a screening tool in early drug discovery. The concept might then be expanded to other ion channels. The ultimate goal of this investigation is therefore to assess the feasibility and translational value of binding information as the basis for establishing *in vitro–in vivo* correlations for drugs with varying affinity for the hERG channel.

Limitations of the assessment of hERG channel blockade

Even though different functional assays are available for screening, the use of *in vitro* hERG inhibition is based on the assumption that any strong signal, that is, hERG channel inhibition, will be predictive of potential QT prolongation *in vivo*. Yet, none of the available preclinical *in vitro* and *in vivo* methods appear to fully predict the torsadogenic potential in humans (Hoffmann and Warner, 2006). Among other things, there has been limited attention to whether experimental conditions are representative of the physiological milieu in humans (e.g. low K^+ concentration, proteins and

low rate stimulation imitating bradycardia). Most importantly, the experiments are performed without taking into account the most likely range of drug exposure at the therapeutic dose levels. To better understand the implications of differences in experimental conditions, a brief overview of the hERG assays and their relevance for the evaluation of pro-arrhythmic effects is provided in the Supporting Information.

Based on the aforementioned hERG channel properties, the use of IC_{50} values characterizing the potential of a compound to block the hERG current provides a convenient way to compare compounds. However, it should be recognized that potency estimates represent an oversimplification of complex time-, voltage- and state-dependent processes. Part of this complexity relates to the fact that some compounds will bind in the open phase of the hERG channel (open state blockers), whereas others will bind when the ion channel is closed again, but the channel needs to be activated first. Also, the association rate for the ion channel has an influence (Yu *et al.*, 2015). Thus, the configuration of the voltage clamp waveform (i.e. the time at a certain voltage and the voltage steps) may affect the potency of the drug as well as the time course of inhibition and recovery, reflecting interactions with different states of the channel.

One of the main implications of such differences hERG binding and inhibition is the high incidence of false positive and false negative results in QT prolongation, as previously illustrated in the publications by Chiang *et al.* (2010), Laverty *et al.* (2011) and Mirams *et al.* (2014); all of which provide figures associated with the sensitivity and specificity of experimental protocols used for drug screening. With regard to false positive results, it should be highlighted that despite a considerable debate supporting the views that hERG inhibition in non-clinical assays are highly predictive of drug effects on the QT interval, exceptions exist, which raise questions about the generalizability of such correlations (e.g. verapamil, which has a high potency for the hERG ion channel but does not prolong the QT interval *in vivo*) (Wallis, 2010). It has been suggested that false positive results are due to the actions of a compound on currents other than the rapid delayed rectifier current (I_{Kr}) such as L-type Ca^{2+} channel current or Na^{+} channel current (Antzelevitch *et al.*, 2004). On the other hand, false negative results in hERG assay data are linked to the fact that various sequentially activated ion channels and transporters may affect the action potential duration. Dumotier *et al.* (2008) suggest that false negatives in hERG inhibition data arise from (i) effects of other ionic currents; (ii) additional effects such as hERG trafficking inhibition; (iii) drug accumulation in the ventricular myocardium; and (iv) drug metabolite effects on hERG current even if parent drug has no effect.

Another important limitation is the questionable accuracy of the so-called quantitative parameters. Usually, IC_{50} values are used to compare and rank compounds. Given the complexities associated with hERG channel inhibition, conclusions drawn from such comparisons may not be accurate. This has potentially important implications for the selection of novel molecules. The bias caused by intrinsic mechanistic differences is often further compounded by other sources of variability in experimental protocols. For instance, temperature has been shown to affect IC_{50} values for some compounds (Kirsch *et al.*, 2004; Yao *et al.*, 2005). Differences in experimental procedures and techniques contribute to the range of IC_{50} values reported for any given compound in the literature. These discrepancies indicate that a relative bias remains for different protocol settings even when positive control standards are used to monitor assay sensitivity (Su *et al.*, 2006).

In spite of the fact that different chemotypes can bind with high affinity to the hERG channel and changes in the functional group or structure of a given chemotype can alter the hERG binding profile significantly (Sanguinetti and Tristani-Firouzi, 2006; Polak *et al.*, 2009; Eichenbaum *et al.*, 2012), here we explore the notion of target occupancy as a screening parameter with potentially direct clinical meaning. Irrespective of whether a systematic relationship between binding and effect can be identified across compounds, we believe that assessment of the correlation between binding and the degree of hERG channel blockade will shed further light on the relevance of parameter estimates such as IC_{50} arising from functional assays *in vitro*.

Methods

In vitro displacement and functional assays

[^{3}H]-dofetilide-isolated membrane displacement. Dofetilide, moxifloxacin, sotalol and cisapride were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). [^{3}H] Dofetilide (specific activity 70.0 Ci·mmol^{-1}) was purchased from Perkin Elmer (Groningen, The Netherlands). BSA (fraction V) was purchased from Sigma (St. Louis, MO, USA). G418 (geneticin) was obtained from Stratagene (Cedar Creek, USA). All the other chemicals were of analytical grade and obtained from standard commercial sources. HEK293 cells stably expressing the hERG K^+ channel (hERG/HEK293) were kindly provided by Dr. Eckhard Ficker (University of Cleveland, USA).

Cell culture. hERG/HEK293 cells were cultured in a humidified atmosphere at 37°C and 7% CO_2 in DMEM, containing 10% FCS, 50 IU·mL^{-1} penicillin, 50 μg·mL^{-1} streptomycin and 1.25 μg·mL^{-1} G418. Initially, cells were sub cultured twice a week (1:8). Then, the cells were sub cultured 1:10 and transferred to large 15 cm diameter plates for membrane preparation.

Membrane preparation. hERG/HEK293 cells were grown to 80–90% confluence and detached from the plates by scraping them into 5 mL of PBS. Then, the detached cells were collected and centrifuged at 250 *g* for 10 min. The cell pellets were pooled and resuspended in 50 mM ice-cold Tris–HCl buffer containing 2 mM $MgCl_2$, pH 7.4. An UltraTurrax (Heidolph Instruments, Schwabach, Germany) was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100 000 *g* in an Optima LE-80 K ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) at 4°C for 20 min. The pellets were resuspended using similar procedures in ice-cold incubation buffer (10 mM HEPES, 130 mM NaCl, 60 mM KCl, 0.8 mM $MgCl_2$, 1 mM EGTA, 10 mM glucose, 0.1%

BSA, pH 7.4) using the UltraTurrax. Aliquots (125 or 250 μL) were stored at −80°C. The protein concentration of the membranes was measured using the bicinchoninic acid method (Smith *et al.*, 1985).

Equilibrium radio ligand binding assays. The [^{3}H]-dofetilide equilibrium binding assays for the hERG K^+ channel were performed as described previously (Chadwick *et al.*, 1993; Finlayson *et al.*, 2001; Chiu *et al.*, 2004; Diaz *et al.*, 2004) with minor modifications. In short, membrane aliquots containing 20 μg protein were incubated in a total volume of 100 μL incubation buffer at 25°C for 60 min. Radioligand displacement experiments were conducted using a range of concentrations of the competing ligand in the presence of 5 nM [^{3}H]-dofetilide. At this concentration, total radioligand binding did not exceed 10% of the initial radioligand added to prevent the ligand depletion. Non-specific binding was determined in the presence of 10 μM astemizole and represented approximately 15% of the total binding. [^{3}H]-dofetilide did not bind specifically to membranes prepared from empty HEK293 cells lacking the hERG K^+ channel (data not shown). Total binding was determined in the presence of incubation buffer and was set at 100% in all experiments, whereas non-specific binding was set at 0%. Given the scope of the analysis, data were normalized as percentage (%) dissociation in order to ensure direct comparison across experiments and normalize for eventual differences in assay handling. Incubations were terminated by dilution with ice-cold wash buffer. Separation of bound from free radioligand was performed by rapid filtration through a 96-well GF/B filter plate using a Perkin Elmer Filtermate-harvester (Perkin Elmer). Filters were subsequently washed 12 times with ice-cold wash buffer. The filter-bound radioactivity was determined by scintillation spectrometry using the P-E 1450 Microbeta Wallac Trilux scintillation counter (Perkin Elmer) after addition of 25 μL microscint and 2 h extraction. The protocol was based on triplicates (n = 3), which is considered standard practice for these experiments.

Whole cell patch-clamp. hERG patch clamp data from reference compounds cisapride, sotalol and moxifloxacin respectively were retrieved from the TI-Pharma data repository. The hERG assays were all performed using kidney (HEK293) cell lines stably transfected with a (plasmid) expression vector with the cytomegalovirus promoter and a neomycin-resistance marker (pcDNA3) expressing hERG (University of Wisconsin, USA). These cells are fully characterized (Zhou *et al.*, 1998) and are the most widely used cells in functional isolated whole cell patch-clamp hERG assays. The extracellular solution consists of (mM): 150 NaCl; 1.8 $CaCl_2$; 1 $MgCl_2$; 5 glucose; 10 HEPES; at pH 7.4. KCl and assay voltage varied in each study (see Table 1 for details). Most protocols were run at 15 s intervals. The amplitude of the tail current following the voltage step back to baseline was measured relative to the holding potential. The signals were corrected for the averaged rundown observed during approximately 10 min exposure to vehicle solution. All values were given as % of control values for each concentration tested. All experiments were performed at room temperature.

In vivo PKPD studies in conscious dogs

Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath and Lilley, 2015). All experiments were approved by the institutional Ethics Committee and conducted according to the ethical standards and Good Laboratory Practice procedures. Telemetric recordings of the ECG were performed in conscious dogs (Ollerstam *et al.*, 2006; Prior *et al.*, 2009). In brief, dogs were implanted with a telemetric transmitter under general anaesthesia. The transmitter was placed in the peritoneal cavity, and electrodes were placed in lead II configuration. A four-way Latin square crossover design was used for the experiments, during which animals administered vehicle, a sub-therapeutic, a therapeutic and a supra-therapeutic oral dose of each compound followed by a washout period between each treatment. The data were recorded and analysed using the Notocord data acquisition system (HEMsoftware, Notocord Inc., Croissy-sur-Seine, France). The Dataquest Open ART™ software (St. Paul, MN, USA) was then used to set up and calibrate the telemetry systems.

The QT interval duration was measured for every wave complex. The placement of the ECG callipers was checked and manually corrected if deemed necessary. Blood samples for pharmacokinetics were collected at different times after dosing to ensure accurate characterization of the absorption, distribution and elimination phases. ECG was monitored continuously over the period of 24 h and averaged every 30 sec (cisapride), 1 min (moxifloxacin) or 5 min (d,l-sotalol). Further details can be found in Chain and Dubois *et al.* (Chain, Dubois *et al.*, 2013).

Clinical trials in healthy subjects. Data from three Phase I studies were included in this analysis. The studies were conducted in full conformance with the principles of the Declaration of Helsinki and with the local laws and regulations concerning clinical trials. The protocol and the informed consent documents for each study have been formally approved by the relevant research Ethics Committee. For cisapride, a randomized, placebo-controlled, dose-escalating design was used in which subjects received up to five doses. Due to safety issues, the study was not continued after the fourth dose. d, l-Sotalol data was extracted from a double-blind, randomized, placebo-controlled, three-way crossover study in which each subject received one active treatment and two placebo doses. Data for moxifloxacin was available from the positive control arm of a two-way crossover, single-blind, randomized, placebo-controlled trial. Additional information regarding the experimental procedures, including electrode placement and blood sampling for pharmacokinetics, has been previously published and can be found elsewhere (Chain, Dubois *et al.*, 2013).

Data analysis

[^{3}H]-dofetilide-isolated membrane displacement. An I_{max} (maximum inhibitory effect) model was used to describe the displacement of [^{3}H]-dofetilide (equation 1):

$$Displacement = I_0 + \frac{I_{max} - I_0 \times [C]}{IC_{50} + [C]} \quad (1)$$

where *Displacement* represents the degree of [^{3}H]-dofetilide displacement. I_0 is the baseline dofetilide binding, I_{max} the maximum displacement and IC_{50} the concentration at

Table 1

Patch clamp assay data available for modelling

Compound	Data set ID	# of cells tested	Cell line	Temperature	$[K^+]$ (mM)	Voltage protocol steps	Concentration range tested (nM)	IC_{50} observed**
Cisapride	225*	11	HEK293	RT	4	−80 mV, +20 mV 5 s, −50 mV 5 s, −80 mV	0.1, 0.3, 1, 3, 10, 30, 100	6.8
	248	9	HEK293	RT	10	−80 mV, +30 mV 1 s, −80 mV	19.5, 39.1, 156, 313, 625, 1250, 2500, 5000, 10000, 20000	938
	249	4	HEK293	RT	5.3	−80 mV, +30 mV 1 s, −80 mV	3, 10, 30, 100	18
	254	7	HEK293	RT	4	−80 mV, −60 mV 0.5 s, +60 mV 2 s, −40 mV 6 s	10, 100, 1000	65
	256	3	HEK293	RT	4	−80 mV, +20 mV 4 s, −50 mV 3 s, −80 mV 8 s	1, 10, 100	19
Moxifloxacin	240*	17	HEK293	RT	5.4	0 mV, −80 mV 20 ms, +40 mV 80 ms	1000, 10 000, 100 000, 300 000, 1 000 000	353 600
	252*	5	HEK293	RT	5.3	−80 mV, +30 mV 1 s, −80 mV	10 000, 30 000, 100 000, 300 000	141 000
	254*	6	HEK293	RT	4	−80 mV, −60 mV 0.5 s, +40 mV 2 s, −40 mv 6 s	30 000, 100 000, 300 000	122 000
Sotalol	217*	4	HEK293	RT	4	−80 mV, +20 mV 0.5 s, −50 mV 5 s, −80 mV	10 000, 30 000, 100 000, 300 000	163 000
	222*	4	HEK293	RT	4	−80 mV, +20 mV 0.5 s, −50 mV 5 s, −80 mV	10 000, 30 000, 100 000, 300 000	117 000

Data included in the current analysis are marked with an asterisk (*). **For the sake of completeness, IC_{50} values (nM) derived from the original experimental protocols are also presented along with the experimental protocol details. These estimates may differ from the values obtained by nonlinear mixed effects modelling, which was used to analyse the data in the current investigation.

which 50% displacement is observed. C is concentration of compound tested.

In contrast to functional measures, drug binding was expected to provide information about target occupancy or blockade and as such reflect the differences in the affinity of each ligand for the hERG channel. Parameters of interest are expressed as inhibitory concentrations but reflect the degree of [^{3}H]-dofetilide displacement: IC_{20}, IC_{50}, IC_{70} and IC_{80} represent the concentrations associated with 20, 50, 70 and 80% displacement. The concentrations of competing ligands were analysed using the nonlinear regression function in R. Nonlinear mixed effects modelling was not deemed necessary due to the number of samples and limited variability in the results obtained with the proposed protocol design. It should also be highlighted that no statistical hypothesis testing was performed to compare differences between compounds.

Whole cell patch-clamp. A sigmoid I_{max} model was chosen to describe the inhibitory drug effects on hERG channel (equation 2):

$$Inhibition = I_0 + \frac{I_{max} - I_0 \times [C]^{\gamma}}{IC_{50}{}^{\gamma} + [C]^{\gamma}} \quad (2)$$

where *Inhibition* is the effect. I_0 is the baseline inhibition, I_{max} the maximum inhibition and IC_{50} the concentration at which 50% inhibition is observed. C is concentration of compound tested, and γ describes the shape or steepness of the curve.

The analysis was performed using nonlinear mixed effects modelling in NONMEM v.7.1.2 (ICON, MD, USA) running on a Windows PC. Model diagnostics were based on graphical and statistical criteria, including goodness-of-fit plots, visual predictive checks and normalized prediction distribution errors (NPDE). All three compounds were analysed concurrently, yielding common parameter estimates for I_0, and I_{max}. Baseline and maximum inhibition were deemed to be comparable across the compounds and as such reflect the experimental conditions (i.e. system-specific properties). By contrast, drug potency varied for each drug. Additive error terms were estimated separately where appropriate.

In vivo and clinical PKPD studies. Estimates of the effects of cisapride, sotalol and moxifloxacin on QT interval prolongation in dogs and healthy subjects, expressed in terms of the concentrations corresponding to a probability of QT interval prolongation ≥10 ms, were used as a clinical reference for establishing potential correlations between *in vitro* and *in vivo* experiments. Full details of the experimental protocols and approval by the ethics committees can be found elsewhere (Chain, Dubois *et al.*, 2013). The PKPD analysis was performed using a Bayesian hierarchical model in WinBUGS version 1.4.2 (Lunn *et al.*, 2002). The model comprises three components, including an individual correction factor for RR interval (heart rate), an oscillatory component describing the circadian variation and a truncated Emax model, as shown by equation 3:

$$QT = QTc_0 \cdot RR^{\alpha} + A \cdot \cos\left(\frac{2\pi}{24}(t - \phi)\right) + slope \cdot C \quad (3)$$

where QTc_0 [ms] is the intercept of the QT-RR relationship (for each individual), RR [s] is the interval between successive R waves, α is the individual heart rate correction factor, A [ms] is the amplitude of circadian rhythm, t is the clock time, Φ is the phase, slope [ms/concentration unit] is the linear pharmacodynamic relationship and C is the observed or, when not available, predicted concentration of the drug at the time of QT measurements.

One of the advantages of this approach is the possibility of characterizing drug effect in a quantitative manner and expressing it in terms of the probability relative to a clinically relevant threshold, irrespective of the baseline QTc values. For the purposes of our analysis, a threshold of ≥10 ms increase in QT was used to assess the relationship between total plasma concentration and the probability of QT prolongation. This reference threshold was selected due to its clinical relevance with regard to the increased risk of TdP. Moreover, there is some evidence that in humans and dogs, QT prolongation does not seem to correlate with baseline QT after correction for differences in heart rate. It should also be noted that plasma protein binding was assumed not to be restrictive for these compounds. Unbound drug concentrations would have yielded similar results, but different apparent parameter values would have been derived after correction for protein binding.

In vitro–in vivo correlation (IVIVC). The assessment of a potential correlation between binding, functional assay and the probability of QT interval prolongation in dogs and healthy subjects was based on graphical summaries, linear and log-linear regression techniques. To ensure normalization of the results across different compounds and experimental protocols, the intercept of the log-linear regression obtained from the IC_{20}, IC_{50} and IC_{70} values of the [^{3}H]-dofetilide-isolated membrane binding assays was calculated and used as parameters of interest for the *in vitro* experiments. In principle, the intercept of this regression corresponds to the concentrations associated with the initial, detectable onset of displacement of [^{3}H]-dofetilide (Oinh). It was compared with the IC_{50} values obtained from hERG patch clamp assays and concentrations corresponding to 50% probability of QTc prolongation ≥10 ms (i.e. CP50) in dogs and humans. All calculations were performed in R 2.12.1.

Each of the analysis described above was performed in an unblinded manner.

Results

[^{3}H]-dofetilide-isolated membrane binding

All three compounds produced a concentration-dependent displacement of the specific [^{3}H]-dofetilide binding. Even though experimental data were sampled in triplicates, variability was relatively low. The maximum SD was 12.3%, with only three measurement points over 6%. The displacement curves were best described by a maximum inhibitory effect model. An overview of the concentration versus displacement curves for all three compounds is presented in Figure 1 along with the functional assay results and concentration versus probability of QT prolongation ≥10 ms. The model-predicted parameter estimates are listed in Table 2.

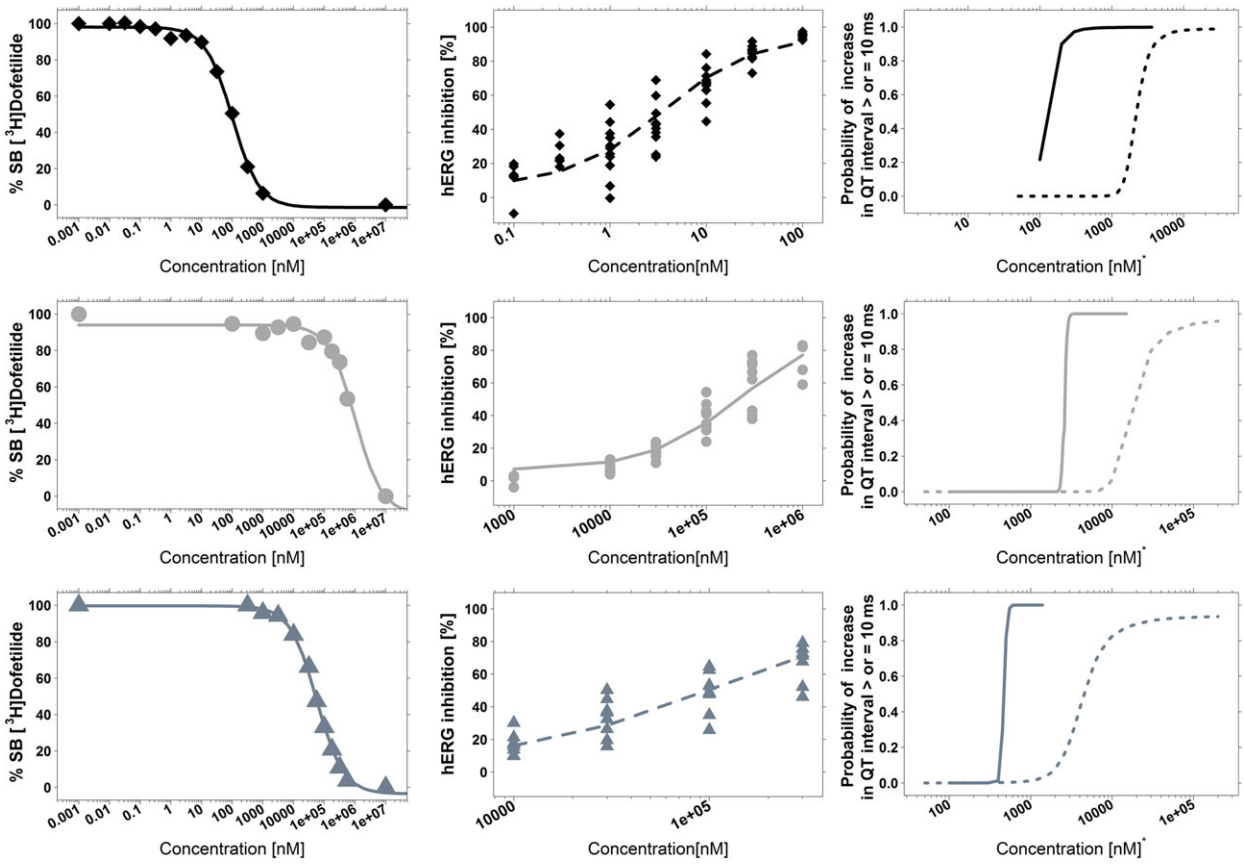

Figure 1

Overview of the concentration–effect relationships derived from *in vitro* (binding vs. functional hERG assay) and *in vivo* data (QT prolongation) in dogs and humans for cisapride (top), moxifloxacin (middle) and sotalol (bottom). Left panels show the dofetilide displacement assay; mid panels display the functional hERG patch clamp assay, whereas the curves depicting the concentration versus probability of reaching ≥10 ms QT prolongation in dogs (dotted line) and humans (solid line) are shown on the right panels (Chain, Dubois *et al.*, 2013). In all plots, the lines represent the model-based population predictions and the symbols the experimental observations. For group size details see Tables 2, 3 and 4.

Table 2

Mean parameter estimates (90% confidence intervals) and derived PKPD indices (IC_{20}, IC_{70} and IC_{80})

	Cisapride	Moxifloxacin	Sotalol
I_0 (%)	98.1 (96.5–99.7)	94.0 (90.9–97.2)	99.7 (97.9–101.6)
I_{max} (%)	99.5 (96.0–103.0)	104 (94.0–114.5)	103 (100.3–106.3)
IC_{50} (μM)	0.1 (0.086–0.12)	1030 (752–1466)	56.4 (50.4–63.2)
IC_{20} (μM)	0.02	160.68	13.32
IC_{70} (μM)	0.22	1649.88	117.18
IC_{80} (μM)	0.36	2543.77	190.81

Data (n = 3 for each experimental point) from the equilibrium [^{3}H]-dofetilide binding displacement assay was analysed using an I_{max} model. I_0 represents the percentage [^{3}H]-dofetilide binding in the absence of a competing molecule. IC_{20}, IC_{70} and IC_{80} are the inhibitory concentrations associated with 20, 70 and 80% binding, respectively.

Cisapride had the highest affinity for the hERG K^+ channel, displacing [^{3}H]-dofetilide with an IC_{50} value of 99.9 nM, whereas moxifloxacin exhibited the lowest affinity of 1030 μM. Sotalol showed displacement with an IC_{50} value of 56.4 μM.

hERG functional assay – whole cell patch clamp

Data from multiple whole cell patch-clamp experiments, including cisapride, sotalol and moxifloxacin, were pooled and explored for consistency and homogeneity before data

fitting. In contrast to the experimental results obtained from the [^{3}H]-dofetilide-isolated membrane binding, functional hERG inhibition was considerably variable. Of note was the variability in the experimental data from cisapride, which had apparent IC_{50} values varying >1000-fold across protocols. The inhibition curves for all three compounds were best described by a sigmoid maximum inhibitory effect model (Figure 1). Goodness-of-fit plots showed that model predictions (population predictions and individual predictions) were able to describe the observed hERG inhibition. Conditional weighted residuals showed no trend for population predictions and only minor trends for individual concentration values. NPDEs were normally distributed with a 0 mean and 1 variance, without a trend (Figures S1 and S2).

Model-predicted IC_{50} values ranged from 3.57 nM for cisapride to 103 μM for sotalol and 227 μM for moxifloxacin. I_0 and I_{max} were found to be around physiologically plausible values, that is, 6.4% and 95.7% respectively. High inter-individual variability (60%) was found for IC_{50} estimates, with a residual additive error of 23.3% for sotalol and moxifloxacin and 57.7% for cisapride (Table 3). The shape parameter γ was close to 1. Despite the satisfactory model diagnostics, predicted individual inhibition curves could not be derived due to the poor precision of the parameters describing inter-individual variability.

In vitro–in vivo correlation

In order to establish whether an *in vitro–in vivo* correlation exists between hERG binding, inhibition and clinically relevant changes in QT interval, the IC_{20}, IC_{50}, IC_{70} and IC_{80} values from the *in vitro* displacement assays (Table 2) and the potency estimates from the functional assay using patch clamp (Table 3) were compared with CP50 estimates previously described by Chain, Dubois *et al*. (2013) (Table 4). For the sake of clarity, the reader is also advised to assess the overall relationship between drug concentration and probability of QT interval ≥10 ms (Figure 1).

Figure 2 shows that the slopes describing the displacement curves for cisapride, sotalol and moxifloxacin are of the same order of magnitude. However, no correlation was found between the concentrations at which displacement occurs (i.e. the beginning of linear portion of the curve) and the predicted CP50 values in dogs and humans. For cisapride, this value occurs at lower values than the CP50 in humans,

Table 3

Population parameter estimates for cisapride, moxifloxacin and sotalol in the hERG patch clamp assay

Parameter	Cisapride (*n* = 11)	Moxifloxacin (*n* = 28)	Sotalol (*n* = 8)
I_0 (%)	6.43*	6.43*	6.43*
I_{max} (%)	95.7*	95.7*	95.7*
IC_{50} (nM)	3.57	227 000	103 000
γ	0.887*	0.887*	0.887*
Interindividual variability (CV%)	60	60	60
Additive error (CV%)	57.7	23.3	23.3

Experimental data obtained with the different compounds were analysed concomitantly. System-specific parameters (I_0, I_{max} and Hill coefficient (γ)) were unique to the experimental setting. Therefore, the values with an asterisk (*) have been estimated only once and are applicable to all three drugs. By contrast, IC_{50} varied for each compound and defined as distinct parameters in the model. A separate additive error term was estimated for cisapride as residual variability in those experiments was significantly higher. CV% indicates the coefficient of variation in percentage.

Table 4

Population PKPD parameter estimates along with 90% credible intervals describing the probability of QT interval prolongation ≥10 ms, as reported by Chain, Dubois *et al.*, 2013

	Cisapride		Moxifloxacin		Sotalol	
Primary and derived model parameters	Dogs (*n* = 8)	Healthy subjects (*n* = 24)	Dogs (*n* = 8)	Healthy subjects (*n* = 137)	Dogs (*n* = 6)	Healthy subjects (*n* = 30)
Slope [ms·nM^{-1}]	0.0045 (0.00096–0.0098)	0.09 (0.087–0.12)	0.00056 (0.00002–0.0014)	0.0039 (0.0033–0.0044)	0.002 (0.0006–0.008)	0.021 (0.017–0.026)
Prob. of ≥10 ms increase at Cmax	0.75	1.0	1.0	1.0	0.9	1.0
Cmax (nM)	2808	936	112 930	10 300	22 310	5605
CP50 (nM)	2200	140	6400	2644	4600	470

Data analysis was performed using a Bayesian hierarchical model, which comprises three components, namely: an individual correction factor for RR interval (heart rate), an oscillatory component describing the circadian variation and a truncated Emax model, which is parameterised in terms of a slope.

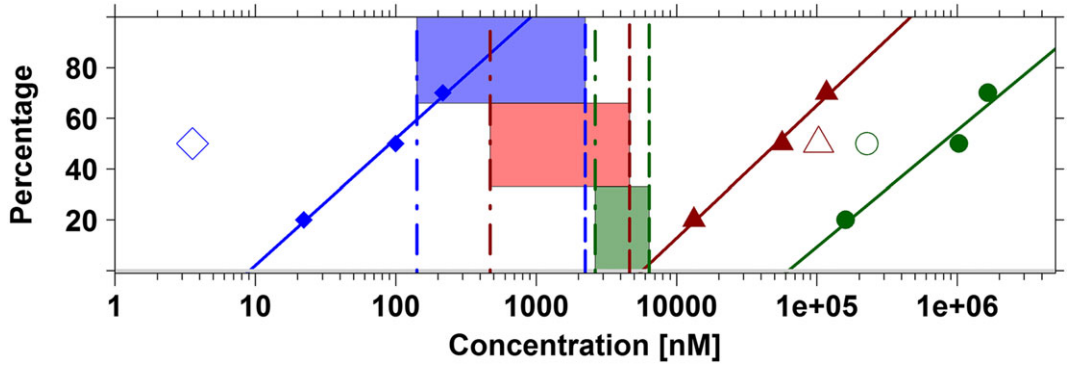

Figure 2

Lack of correlation between *in vitro* (dofetilide displacement, functional assay) and *in vivo* (probabilities of QT prolongation *in vivo* in dogs and humans) data. Ideally, proportional differences should be found between these experiments, reflecting the relative differences in the potency of the different compounds. Data summaries include cisapride (blue diamonds), sotalol (red triangles) and moxifloxacin (dark green dots). Log-linear regression (solid lines) of the estimates of IC_{20}, IC_{50} and IC_{70} obtained from the dofetilide displacement are compared with the IC_{50} values obtained from hERG patch clamp assays (open symbols) and *in vivo* (dashed vertical lines) and clinical (dot-dashed vertical lines) concentrations corresponding to 50% probability of QTc prolongation ≥10 ms (i.e. CP50). The window associated with these concentrations (i.e. CP50) in dogs and humans is depicted using the blue, red and dark green shaded areas for cisapride, sotalol and moxifloxacin, respectively. For instance, for cisapride, IC_{50} in the binding assay indicates a potency of 99.9 nM. In contrast, potency in the hERG functional assay is much higher, that is, 3.57 nM. These values contrast with CP50 estimate *in vivo* in dogs and in healthy subjects, which yield estimates of 2233.6 and 141.5 nM, respectively. See Table 5 for further details. Parameters describing the log-linear regression for cisapride, sotalol and moxifloxacin were respectively $y = -49.85 + 49.91^{*}\log(x)$, $y = -196.08 + 52.22^{*}\log(x)$ and $y = -220.46 + 45.96^{*}\log(x)$.

whereas for moxifloxacin and sotalol the onset of displacement occurs at values higher than the CP50 in dogs. Similarly, there was no clear pattern or correlation between IC_{50} estimates derived from hERG patch clamp and CP50 in dogs and humans. The IC_{50} of cisapride was around 40-fold lower than the CP50 in humans, whereas sotalol and moxifloxacin have >80-fold or >20-fold difference compared with the CP50 in humans and dogs, respectively. An overview of the parameters used to establish a potential *in vitro–in vivo* correlation is presented in Table 5.

Table 5

Summary table showing the comparison of the estimates obtained by the analysis of *in vitro* (dofetilide displacement, functional assay) and *in vivo* (probabilities of QT prolongation *in vivo* in dogs and humans) data

	Cisapride	Moxifloxacin	Sotalol
CP50D (μM)	2.23	6.39	4.62
CP50H (μM)	0.14	2.64	0.47
Oinh (μM)	0.09992	62.62	5.69
Ratio Oinh/CP50D	0.004	9.8	1.2
Ratio Oinh/CP50H	0.07	23.7	12.1
IC_{50} (μM)	0.00357	227	103
Ratio IC_{50}/CP50D	0.0016	35.53	22.3
Ratio IC_{50}/CP50H	0.026	86.0	219.1

The onset of inhibition (Oinh) was obtained by log-linear regression using mean IC_{20}, IC_{50} and IC_{70} estimates from the displacement assays. IC_{50} values are population parameter estimates obtained by nonlinear mixed effects modelling of the patch clamp assays. CP50D and CP50H are the concentrations associated with a 50% probability of reaching ≥10 ms increase in the QT interval in dogs and humans, respectively.

Discussion

Functional inhibition of the hERG channel assays have been used systematically in drug development since the discovery of a link between drug-induced hERG inhibition and TdP. Typically, IC_{50} values in μM or nM range are compared with projected plasma concentrations to define a safety margin; that is, the closer these values get the more the results are considered as a liability for QT interval prolongation in humans. As a screening tool, these experiments have evolved to show high sensitivity to changes in hERG function, irrespective of growing evidence about the differences between the degree of inhibition, QT prolongation and TdP (Wallis, 2010; Di Veroli *et al.*, 2014).

This situation is not unique to the screening of candidate molecules and often reflects common practice in experimental safety protocols that are used after candidate selection (Sahota *et al.*, 2015). In addition to the lack of standardization for experimental procedures, a general feature in these protocols is the absence of information regarding the underlying concentration–effect relationships, which can be used as a denominator across experimental conditions and species. Our own group has shown that PKPD relationships can be used to establish in a quantitative manner how changes in drug exposure relate to QT/QTc interval prolongation in preclinical species and in humans. Moreover, we have shown that by using the appropriate parameterization, it is possible to distinguish between drug- and system-specific properties, making it clear how different drugs relate to each other. In fact, a model-based approach may allow one to disentangle

pharmacokinetic from pharmacodynamic differences as well as other intrinsic or extrinsic factors contributing to variability in drug effects (Danhof *et al.*, 2008).

Therefore, the current investigation was aimed at exploring whether binding and functional inhibition could be linked to QTc interval prolongation in preclinical species and in humans using PKPD modelling. Evidence of an *in vitro–in vivo* correlation for compounds with known QT prolonging effects may provide further insight into the role of differences in receptor density and binding kinetics and consequently facilitate the translation of early findings (Della Pasqua, 2013; France and Della Pasqua, 2015). In addition, evidence of such a correlation might provide the basis for experimental protocol standardization, reducing the rate of false positive and false negative results, for which accurate figures are variable due to differences in experimental protocols and by the discontinuation of compounds (i.e. no clinical data are available for compounds with strong preclinical signals). This problem is illustrated by the case of verapamil, which shows a significant hERG signal, but does not produce QT interval prolongation (Chiang *et al.*, 2010; Laverty *et al.*, 2011; Mirams *et al.*, 2014). If verapamil were developed according to current screening criteria, it might have been discontinued before reaching the clinic.

Clearly, our results contrast with previous publications in which a claim has been made about the predictive performance of the hERG functional assay (Gintant *et al.*, 2006; Wallis, 2010). However, it should be noted that in most investigations data have not been generated or compared in a systematic manner using a model-based approach. As can be seen from the summary results in Figure 2, it appears that not only the concentration at which effects become evident (i.e. onset of inhibition) varies between compounds but also the relationship between binding and hERG inhibition. Moreover, these inflection points do not correlate with the predicted CP50 values in humans (i.e. Oinh vs. CP50 in Table 5).

Furthermore, it appears that the variability in hERG patch clamp data is large and very sensitive to differences in experimental protocol conditions. Such variability affects the potency estimates and may lead to inaccurate ranking of candidate molecules when comparing functional assay results. Most importantly, potency estimates from whole cell patch clamp do not appear to be strictly predictive of the drug levels associated with QT/QTc interval prolongation in dogs and humans even if one takes into account the potential role of differences in plasma protein binding. Similar conclusions were also drawn by Watson *et al.* (2011) when comparing modelling results in cynomolgus monkeys with the concentration–response relationships of hERG current derived from *in vitro* patch clamp experiments.

Given this mismatch between functional measures *in vitro* and clinical effects, it would be of interest to establish whether binding information, as assessed by [H^3]-dofetilide displacement, bears any correlation with QT interval prolongation *in vivo*. Surprisingly, this turns out not to be the case, at least for these three compounds. Parameter estimates describing drug affinity for the hERG channel show that displacement occurs at levels, which are much higher than the concentrations associated with QT prolongation ≥10 ms in humans. Furthermore, maximum displacement occurs at very high concentrations, which may represent an important limitation for compounds with poor solubility.

A number of factors may explain these results. First, one should not ignore the fact that hERG ion-channels are overexpressed in HEK293 cells, which may cause a significant increase in the absolute amount of ligand required to block the available pool of binding sites, yielding apparent estimates, which are unlikely to reflect *in vivo* conditions. Second, biophase equilibration kinetics and differences in drug-ion channel interaction may lead to variable signal transduction, yielding different results in functional assays and subsequently discrepancies in action potential duration and QT interval prolongation (Di Veroli *et al.*, 2014; Mirams *et al.*, 2014). Indeed, examples exist in the published literature, which illustrate the implications of delayed equilibration and slow drug-receptor interactions for the pharmacological effect versus time profile of a compound *in vivo* and *in silico* conditions (Yassen *et al.*, 2005; Durdagi *et al.*, 2012; Lee *et al.*, 2016). Another important point to consider explaining the observed discrepancies between *in vitro* and *in vivo* experimental data is that hERG blockade is one of a range of factors associated with pro-arrhythmia. Even though all three compounds are known to bind to hERG channels, it is clearly not the only mechanism underlying drug effects *in vivo*, as for instance in the case of cisapride, for which other ion channels are known to be involved in the observed QT prolonging effect in dogs and humans (Jonsson *et al.*, 2012). Thus, an evaluation of the potential pro-arrhythmic effects of drugs based on hERG binding or current block alone may provide an incomplete view of a drug's effects on cardiac repolarization processes.

In summary, it seems that even when considering concentration–effect relationships, there remains a translational gap between drug screening and QT prolongation in the clinic. It appears that screening and ranking procedures based on binding or potency estimates for inhibitory activity on single ion channels may be misleading, even for compounds with known activity on a single ion channel. Our endeavour to discriminate system- versus drug-specific properties in a parametric manner has not yielded the expected results, in that overall measures of target occupancy or inhibition *in vitro* do not seem to reflect or predict in a quantitative manner the magnitude of drug effects *in vivo* in dogs or in humans.

Undoubtedly, integrative approaches are needed that account for the multifactorial nature of the pro-arrhythmic effects *in vivo*. Among the options available, one should consider the use of a virtual population generator for human cardiomyocyte parameters, as proposed by Polak *et al.* (2012). The authors propose a computational system including simulations for the evaluation of proarrthymic potential (Tusscher *et al.*, 2004; O'Hara *et al.*, 2011), taking into account the influence of inter-individual variability in the parameters of interest. In fact, this concept has been recently used to predict the effects of domperidone (Mishra *et al.*, 2014), illustrating how *in vitro* and more specifically *in silico* simulations can be used in conjunction with physiologically-based pharmacokinetic models to predict drug effects in humans. Despite these promising results, some obvious limitations exist, which cannot be overlooked. None of the *in silico* models currently available account for the contribution of

physiological factors such as body temperature, insulin/glucose homeostasis, changes in electrolytes or autonomic tone known to alter QT interval *in vivo* (Fossa, 2008; van der Linde *et al.*, 2008). These models also ignore the contribution of differences in binding properties and biophase kinetics (Durdagi *et al.*, 2012; Di Veroli *et al.*, 2014; Lee *et al.*, 2016). A comparable situation applies to the extrapolation of QT prolongation from controlled clinical trials to a real-life setting (Chain *et al.*, 2013). This should be carefully considered by those supporting the CiPA working groups, who are currently responsible for the development and implementation of alternative guidelines for ICH S7B, which will ultimately guide the ranking of compounds in terms of their pro-arrhythmic risk (Cavero and Holzgrefe, 2015).

We acknowledge a few limitations in our research. First, it is worth reminding the reader that PKPD parameters should be independent from and uncorrelated with the dose and/or experimental protocol design. We have analysed functional hERG assay data available to the TIPharma consortium, which included contributions from five large pharmaceutical companies. However, we cannot exclude the possibility that different protocol settings might have yielded different results. Despite the different protocols (Table 1), it remains unclear whether they represent the optimal experimental conditions for establishing *in vitro–in vivo* correlations.

Also, others have shown some degree of intra-lab variability (particularly over time) and inter-lab variability even when using the same protocols. The ongoing CiPA project is working closely with contributing scientists and vendors to establish standardized protocols for each/all of the ion channel assays under consideration. Similarly, it should be noted that the variability between *in vivo* pharmacokinetics and pharmacodynamics may contribute to potential inaccuracies in parameter estimates. In this respect, one should be aware that the use of nonlinear mixed effects modelling can take into account the effect of interindividual differences in pharmacokinetics and pharmacodynamics. Moreover, it allows for the discrimination of covariate effects from random variation.

Whereas it is evident that concentration–effect curves and IC_{50} values do not provide information on binding kinetics, PKPD modelling of these relationships may account for differences in the interaction between drugs and ion channels. Appropriate parameterization and specific sampling requirements would apply to ensure identification of association/dissociation rate constants. Unfortunately, the available experimental data were not suitable for such an approach. We anticipate that a similar mismatch between *in vitro* binding, functional assay and QT prolongation would have been observed if other PKPD indices were used, for example IC_{10}, or IC_{20}. A first (or even second) order rate constant may be required to describe biophase equilibration processes.

We are also aware of the potential implication of differences in plasma protein binding for the characterization of PKPD relationships. In fact, controversy regarding the correction for protein binding has been highlighted in previous publications (Gintant *et al.*, 2006). Nevertheless, protein binding for the compounds under evaluation is known to be non-restrictive; that is, the affinity of a compound for the plasma protein does not necessarily alter binding equilibrium relative to the hERG channel or other relevant target channel. We have summarized our results in terms of total concentration as conclusions would not have been different even after correcting for differences in protein binding. It should be noted that the compound with the highest protein binding (i.e. cisapride) also has very high affinity for the hERG channel.

Lastly, we recognize that the results from three reference compounds are not sufficient to allow generalization of the conclusions to a wide class of molecules, as it can be anticipated that changes in a functional group of a given chemotype can alter the hERG binding profile significantly (Polak *et al.*, 2009).

In conclusion, sensitivity and selectivity criteria have driven the development of experimental protocols for the screening of the pro-arrhythmic potential of candidate molecules in early drug discovery. Whilst our investigation is limited to three reference compounds with known QT-prolonging effects in humans, the apparent potency obtained from *in vitro* assays was far higher than the estimates observed *in vivo* for the same drugs. The progression of molecules may be unintentionally stopped due to the discrepancies or lack of a systematic *in vitro–in vivo* correlation. Despite the emphasis on the importance of these assays as a screening tool, our findings indicate that results from *in vitro* protocols are qualitative at best and cannot be used to define the probability of a clinically relevant increase in the QTc interval.

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Author contributions

V.F.S.D. performed the data analysis and wrote the manuscript. E.C. contributed to the analysis of hERG functional assay data. M.D. contributed to the research proposal and revision of the manuscript. O.D.P contributed to the research proposal, data analysis and revision of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 Goodness-of-fit plots. Upper panels show the individual (left) and population (right) predictions versus observed inhibition in the whole cell patch clamp assay. Lower panels show the conditional weighted residuals versus population predicted inhibition values (left) and drug concentration (right). Symbols indicate the different compounds, namely cisapride, sotalol and moxifloxacin.

Figure S2 NPDE summaries for the pharmacokinetic–pharmacodynamic model describing hERG inhibition in the whole cell patch clamp assay. Upper panels show the QQ-plot of the distribution of the NPDEs for a theoretical N (0, 1) distribution (left) and the histogram of the distribution of the NPDE together with the density of the standard normal distribution (right). Lower panels show the NPDEs versus concentrations (left) and NPDEs versus individual predictions (right).